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This report describes the cloning, sequencing and properties of a potential TGFα-releasing protease present in human breast epithelial cells. Degenerate oligonucleotides based on the conserved sequences about the his^{57} and ser^{195} residues in mammalian serine proteases were used to clone a novel serine protease sequence (PCR-7) from an MCF-7 breast cancer cell cDNA library. The deduced 321 amino acid open reading frame shows a high degree of homology with the cell-surface serine proteases enterokinase, prekalikrein, hepsin and prostasin. Northern blot analysis, using a PCR-7 cDNA probe, shows the presence of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells. Similar mRNA species were found in normal breast tissue, trophoblast, and uterine glandular epithelium but not in human U373MG glioma or HepG2 hepatoma cells. In MCF-7 cells, expression of the PCR-7 mRNA was not significantly stimulated by estradiol or TGFα, but was stimulated by IL-6 (200%) and IL-1 (300%). A GST fusion protein constructed from a PCR-7 cDNA sequence was used to raise antibodies in rabbits. On western blot analysis, the antibody recognized a 33 kDa polypeptide in trophoblast and MCF-7 cells under both reducing and non-reducing conditions. This antibody inhibited TPA-induced TGFα release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera.

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CLONING OF A POTENTIAL TGFα-RELEASING SERINE PROTEASE FROM MCF-7 HUMAN BREAST CANCER CELLS

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INTRODUCTION

The broad aim of this proposal is to test the novel hypothesis that the coordinate action of proteases and protease inhibitors are responsible for growth factor activation and release in the human breast cancer cell, and for the progression of breast cancer *in vivo*. One-third of all cases of advanced breast cancer are estrogen responsive, and recent epidemiological studies and studies using human breast cancer cells in culture strongly suggest a correlation between estrogens and the pathogenesis of breast cancer (Harris et al 1992). The mechanisms by which this occurs are not entirely clear. Certainly, estrogens have a direct effect on cell growth. In addition, they can stimulate the expression and release of a variety of polypeptide growth factors. It is highly likely that the tumorigenic effects of estrogens are due, at least in part, to the autocrine/paracrine action of these factors. Several of these polypeptides, including epidermal growth factor (EGF), and its analogs heregulin and transforming growth factor-α (TGFα) and the insulin-like growth factors (IGF-I and IGF-II), have been shown to require pericellular proteolysis for activation or release (Massagué and Pandiella 1993). To achieve homeostasis in a normal breast epithelial cell, levels of these pericellular growth factor activating proteases also must be regulated. We hypothesized that this was accomplished by the action of locally synthesized protease inhibitors. Thus an imbalance in the ratio between local levels of particular proteases and protease inhibitors could be responsible for increases in tumorigenic potential.

TGFα, a peptide structurally and functionally related to EGF, interacts with the EGF receptor and elicits a mitogenic response in a variety of cells (Lee et al 1995). TGFα expression occurs in normal breast tissue, breast tumors and breast cancer cells in culture and TGFα has been proposed to act as a major autocrine mediator of estrogen-stimulated growth in estrogen-dependent breast cancer cells (Harris et al 1992). TGFα is synthesized as part of a glycosylated transmembrane precursor (proTGFα) which can be processed intracellularly and extracellularly by glcosylation and proteolysis to yield a family of polypeptides ranging in size from the 29kDa transmembrane proTGFα to the 6 kDa soluble, mature form (Massagué and Pandiella 1993). Complete processing does not occur to the same extent in all tissues. While all

TGFα forms appear to possess some degree of biological activity, there is good evidence that particular biological actions may depend on the degree of proteolytic processing. The proteolytic cleavage required for release of soluble TGFα from its membrane-bound precursor on the cell surface may be an essential step in regulating TGFα activity.

Results from this laboratory have clearly demonstrated a relationship between tumorigenicity of MCF-7 human breast cancer cells (as measured by growth in soft agar), endogenous synthesis of the protease inhibitor α_1-antitrypsin (α_1-AT) and release of TGFα (Tamir et al 1990, Finlay et al 1993a,b). Growth in soft agar was blocked by α_1-AT whether added to the tissue culture media or synthesized by the tumor cell itself. A useful tool in these studies was a new MCF-7 cell subline, producing 10-fold higher levels of α_1-AT than its parental cell line, constructed by stable transfection with an α_1-AT cDNA (Yavelow et al 1997). Growth in soft agar and release of TGFα was decreased in cells transfected with the α_1-AT cDNA when compared to cells transfected with vector alone. Consistent with the above we had identified a serine protease with elastase-like activity, capable of forming a stable complex with α_1-AT, on the MCF-7 cell surface. In the work described here, we have focused our attention on the identification of the pericellular protease responsible for TGFα release, as this might be a convenient target for anticancer drug action.

METHODS AND MATERIALS

Preparation of cDNA - Total cellular RNA was prepared from MCF-7 (ML) cells by extraction in guanidinium isothiocyanate and centrifugation on cesium chloride. mRNA was isolated using PolyATTract mRNA isolation system IV (Promega) and used to synthesize double stranded cDNA (BRL cDNA synthesis system).

Primers - Degenerate oligonucleotide primers designed from the conserved amino acids around the active site residues his ^{57} and ser^{195} and the oligonucleotide probe based on the conserved sequence around asp ^{102} were synthesized as previously described (Sakanari et al, 1989; Elvin et al, 1993).

PCR conditions - 100 ng of cDNA was amplified in a Perkin Elmer thermal cycler 480 as described by Elvin, et al except that 4 mM $MgCl_2$ was used in the reaction.

Cloning, Southern blotting and sequencing of PCR products - 2μl of the PCR products were ligated directly into the TA cloning vector pCR™II (Invitrogen). 1 μl of the ligation reaction was used to transform E. Coli strain INVαF' (Invitrogen). Recombinant clones were selected by blue / white color selection on LB agar plates containing ampicillin (50μg/ml) and X-gal. Plasmid DNA was isolated from 22 white colonies by alkaline lysis, digested with Eco RI and electrophoresed on a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. After denaturation and neutralization the gel was transferred to

GeneScreen nylon membrane (NEN), baked at 80° C for two hours and hybridized overnight at 37° C with the degenerate Asp^{102} probe end labeled with γ-^{32}P ATP using T4 polynucleotide kinase (Promega). The blot was washed with 4 X SSC, 0.1% SDS for 2 X 5 min at room temperature followed by 40 min at 37° and briefly at room temperature. One strongly hybridizing fragment of ≈ 460 bp, (PCR-7), was gel purified and used as a probe for northern blotting. Plasmids were sequenced using SP6 and T7 promoter primers and Sequenase DNA sequencing kit (USB).

Northern Blots - Total RNA, isolated from MCF-7 cells, U373MG glioma cells, Hep G2 cells, normal human breast tissue, trophoblast and uterine glands was electrophoresed on 1.5% agarose/formaldehyde gels, transferred to GeneScreen and hybridized with ^{32}P-labeled probe. All probes used for northern blots were labeled by Megaprime DNA labeling system (Amersham).

Preparation of cDNA library - Total RNA was extracted from MCF-7 cells using Ultraspec RNA isolation system (Biotecx). mRNA was isolated using the MGP mRNA Purification Kit (CPG). Starting with 3.8 µg of mRNA, a cDNA library was constructed in the plasmid vector pSport 1 using the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Life Technologies) according to the manufacturers instructions. The library was constructed directionally in the Not I-Sal I cut vector such that cloned genes can be expressed *in vitro* using the T7 RNA polymerase promoter. After transformation of E. Coli DH5α the library was amplified on selective agar plates, aliquotted and stored at -70° C.

Screening of cDNA library. Approximately 60,000 recombinant colonies were screened by standard hybridization techniques after transfer to Nytran nylon membranes (Schleicher & Schuell). In order to eliminate any potential background hybridization to plasmid sequences, the 460bp protease insert was gel purified and used as a template for PCR. The primers, determined from the sequence data, amplified a 300bp fragment which was purified and used to probe the library. This probe does not hybridize to plasmid DNA as determined by Southern blot. Positive colonies were selected and rescreened. Insert size was determined by electrophoresis of Not I - Sal I cut plasmid DNA.

Preparation of Fusion Protein and Antibody Production. The 460bp PCR-7 protease fragment was ligated to the Eco RI site of the glutathione S-transferase gene fusion vector pGEX-4T-1 (Pharmacia). The resulting construct as well as the parent vector were used to transform E. Coli strain INVαF', a strain recommended for cloning and maintenance of the plasmids. Selected colonies were grown in liquid culture for 2.5 h at 37° then induced with 0.1mM isopropyl-β-D-thiogalactoside (IPTG) for an additional hour. Cell pellets were suspended in ice-cold PBS, sonicated briefly and electrophoresed on a 12% SDS polyacrylamide gel. After determining which clone was in the right orientation for expression of the GST-PCR-7 fusion protein, the plasmid (pGEX-4T-1-PCR-7) was used to transform E. Coli strain BL21 for large-scale

expression. Fusion protein was purified from 250 ml of an induced culture by the procedure described by Frangioni (1993) for insoluble fusion proteins. Polyclonal antibodies were produced in rabbits by the Pocono Rabbit Farm & Laboratory, Inc. IgG was purified by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-cellulose. Western blots were visualized by autoradiography after detection with ^{125}I protein A.

RESULTS AND DISCUSSION

Taking advantage of the high degree of homology in the DNA sequences about the active site histidine, aspartate and serine residues (his^{57}, asp^{102}, ser^{195}) in all serine proteases, we constructed degenerate oligonucleotide primers which were used to "fish out" putative serine protease sequences from an MCF-7 cell cDNA library. In the initial step, a series of approximately 500 bp fragments between the his^{57} and ser^{195} sites were amplified by PCR. The amplified sequences were then cloned into a PCR cloning vector, which was used to construct a mini-cDNA library. Clones from the mini library were selected by Southern blotting using a ^{32}P-labeled degenerate oligonucleotide probe based on the sequence about asp^{102}. Several positive clones (Fig. 1) were sequenced. The 460 bp sequence of one strongly hybridizing clone, PCR-7, showed a high degree of homology to known serine proteases (64% identity in a 220 bp region at the 3' terminus to human trypsinogen-B and a 60% identity in a 120 bp region around the 5' terminus to human pancreatic protease).

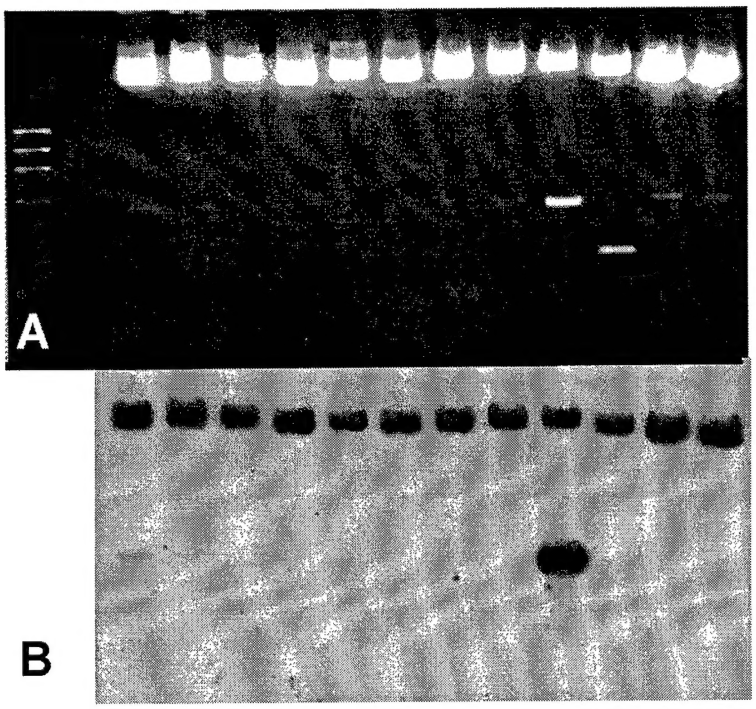

Figure 1. Cloning of MCF-7 Serine Protease Fragments. Putative cDNA inserts corresponding to the his^{57}-ser^{195} sequence were excised from the pcRII cloning vectors, electrophoresed on agarose gels and stained with ethidium bromide (panel A). The DNA fragments were then transferred to nitrocellulose and probed with a ^{32}P-labeled oligonucleotide corresponding to the sequence about asp^{102} (panel B). While all of the clones contained inserts between 400 and 600 bp, in this series only the clone-7 insert (4th lane from right) showed significant hybridization to the asp^{102} probe.

Northern blot analysis, using a PCR-7 cDNA probe, showed the presence of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells. This probe also recognized similar mRNA species in normal breast tissue, trophoblast, and proliferative phase uterine glandular epithelium but not in human U373MG glioma or HepG2 hepatoma cells (Fig. 2). In trophoblast isolated from term placenta, expression of the PCR-7 mRNA was repressed when trophoblast was maintained under hypoxia for 48 h. We have shown that under these conditions, TGFα release is reduced. In MCF-7 cells, expression of the PCR-7 mRNA was not significantly stimulated by estradiol or TGFα, but was stimulated by TGFβ (150%), IL-6 (200%) and IL-1 (300%) (Fig. 3).

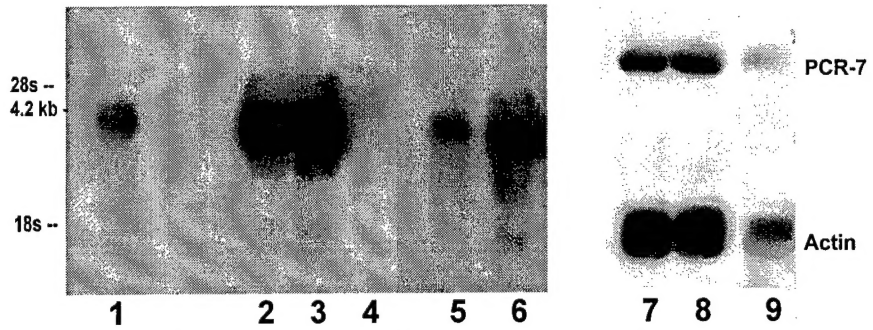

Figure 2. Northern Blot Analysis Using Clone-7 cDNA Probe. Total RNA was prepared from various human tissues and cell lines, electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to GeneScreen nylon membrane and probed with the ^{32}P-labelled cDNA insert. The RNAs shown are lane 1, human breast ductal epithelium; lane 2, MCF-7 ML cells; lane 3, MCF-7 P203 cells; lane 4, U373MG glioma cells; lane 5, term trophoblast cultured under hypoxic conditions; lane 6, term trophoblast cultured under normoxic conditions; lane 7, MCF-7 cells in media containing 10^{-8} M estradiol, lane 8, MCF-7 cells in estradiol-free media; lane 9, uterine glandular epithelium.

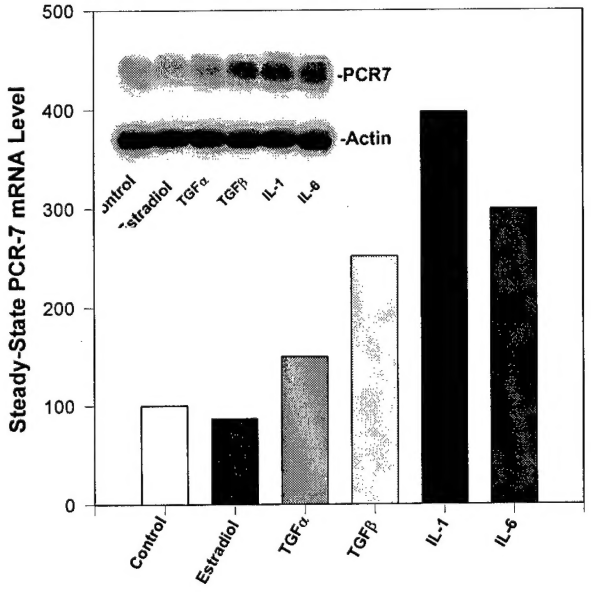

Figure 3. Northern Blot Analysis of MCF-7 Cell RNA. Effect of Growth Factors and Cytokines on PCR-7 mRNA Expression. MCF-7 cells were maintained 48 hours in phenol red-free DMEM containing 10% stripped FBS. The media was replaced with fresh media containing the indicated additions and incubated 24 hours longer. Total RNA (ca. 15 µg) from each sample was subjected to Northern blot analysis with a ^{32}P-labeled PCR-7 cDNA. The blot was reprobed with an actin cDNA. lane 1, no additions; lane 2, 10^{-8} M Estradiol; lane 3, 50 ng/ml TGF-α; lane 4, 5 ng/ml TGF-β; lane 5, 10 u/ml IL-1; lane 6, 200 u/ml IL-6. The blot was reprobed with a ^{32}P-labeled actin cDNA (insert). The blot was densitometrically scanned and integrated

The 460 bp PCR-7 cDNA was also ligated into the glutathione S-transferase (GST) gene fusion vector, pGEX-4T-1. This construct and the parental vector were used to transform E. coli and fusion protein expression was induced with IPTG. On SDS PAGE, 3 of 6 recombinant vectors displayed a prominent band of about 40 kDa representing the GST- PCR-7 fusion protein while the parental vector produced a prominent 29 kDa band characteristic of GST alone. Large scale cultures of bacteria containing the GST- PCR-7 fusion protein vector were prepared and substantial amounts of the GST- PCR-7 fusion protein was isolated. The fusion protein was used to raise antibodies in two rabbits. Antiserum from one of the rabbits was able to

immunoprecipitate the ^{35}S- labeled proteins prepared in vitro described below. Preliminary evidence suggests that this antibody is able to inhibit TPA-induced TGFα release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera (Fig. 4).

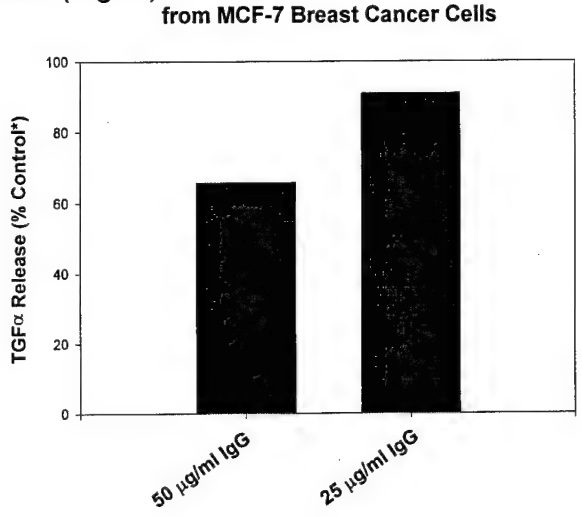

Figure 4. Antibody to PCR-7 fusion protein blocks release of TGFα from MCF-7 cells. MCF-7 (ML) cells were maintained in phenol red - free DMEM containing 10% FBS. Estradiol (10^{-8} M) was added for 24 hours. Cells were then washed twice with serum-free media and allowed to condition serum-free DMEM containing estradiol and IgG for 30 min. TPA (50 ng/ml) was added and the cells were incubated for an additional 4 hours. Conditioned media were concentrated on C_{18} SEP-columns equilibrated with 10% acetonitrile-0.1% TFA eluting with 40% acetonitrile-0.1% TFA. Samples were taken to dryness and resuspended in PBS. TGFα levels were determined using a TGFα ELISA assay (Oncogene Science) according to the manufacturer's instructions. Results are reported as the percentage of TGFα released in the presence of antibody (IgG) to the PCR-7-GST fusion protein as compared to the same level of pre-immune IgG.

Two cDNAs of approximately 3.8 and 3.2 kb containing the PCR-7 sequence were cloned from a second MCF-7 cell cDNA library using the PCR-7 sequence as a probe. The library had been constructed by directional cloning into the multiple cloning site of a pSport I vector such that the 5' terminus was downstream from a T7 promoter sequence. Except for minor differences in the 3' sequence, the two clones appear to be identical where they overlap. Both clones hybridized to the same size mRNA as did the PCR-7 sequence used to screen the library (Fig. 5). Approximately 2000 bp (plus an additional 500 bp at the 5' end) of the larger clone (Clone 1) has been sequenced (Fig. 6). The sequence has a 963 b open reading frame (bases 463-1426) and an approximately 500 b 3' sequence containing the poly adenylation site. The deduced amino acid sequence from the open reading frame contains the His^{57} and Ser^{195} motifs characteristic of serine proteases (amino acid residues 119-124 and 265-275 respectively). The aspartate residue corresponding to Asp^{102}, the third amino acid in the serine protease catalytic triad, is most likely one of the three Asp residues between residues 171 and 177. With BLAST, a high degree of identity at the amino acid level was found between the PCR-7 protease sequence and the serine proteases (or their zymogens) enterokinase precursor (Kitamoto et al 1995), hepsin (Leytus et al 1988), prekalikrein (Chung et al 1986), coagulation factor XI (Fujikawa et al 1986), acrosin precursor (Baba et al 1989) and prostasin (Yu et al 1994, 1995) (Table I). Significantly, enterokinase precursor, hepsin, prekalikrein, acrosin precursor and prostasin are cell surface bound. A high degree of homology was found at the nucleotide level with another cell surface protease, hepatocyte growth factor activator (Miyazawa et al 1993).

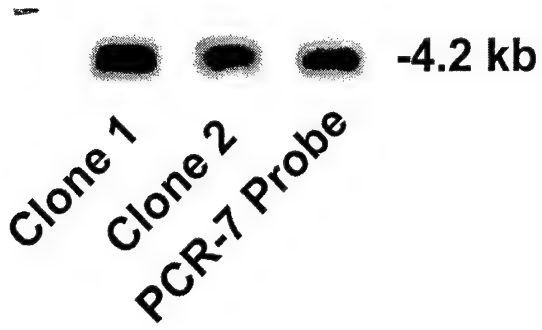

Figure 5. Full Length cDNAs (Clone 1, Clone 2) and 300 bp PCR Fragment Used for Cloning Bind to the Same Size mRNA. The 3.8 (Clone 1) and 3.2 (Clone 2) kb cDNAs containing the PCR-7 sequence were gel purified, labeled with ^{32}P-dCTP and used to probe a northern blot containing 15 µg of ML total RNA / lane. The 300 bp PCR-7 cDNA probe used to screen the library was used as a control.

Table 1. Sequence comparison analysis of PCR-7 Protease with other human serine proteases. Analysis was performed at the NCBI using the BLAST network service.

Protein	% Identity	Overlap
Enterokinase Precursor	40.6	254 aa
Hepsin	39.6	270 aa
Prekallikrein	38.8	260 aa
Factor XI	35.8	327 aa
Acrosin Precursor	35.5	276 aa
Prostasin Precursor	51.9	187 aa
Hepatocyte Growth Factor Activator	53.5	620 bp (740-1360)

Figure 6. Sequence of PCR-7 Protease cDNA. Clones were sequenced at the NYU/Skirball sequencing facility using an Applied Biosystems 373 automated DNA sequencer. Sequencing primers were obtained from Gibco BRL. Deduced sequence of the 321 amino acid open reading frame (bases 463-1426) is shown beneath the corresponding trinucleotide.

```
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1321---------+---------+---------+---------+---------+---------+
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1381---------+---------+---------+---------+---------+---------+
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1921---------+---------+---------+---------+--------
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Trypsin His57 Motif: **ValSerAlaAlaHisCys**
119 124

Trypsin Ser195 Motif: **AspSerCysGlnGlyAspSerGlyGlyProLeu**
265 275

Asn Glycosylation Motif: **AsnGlnThrThr**
241 244

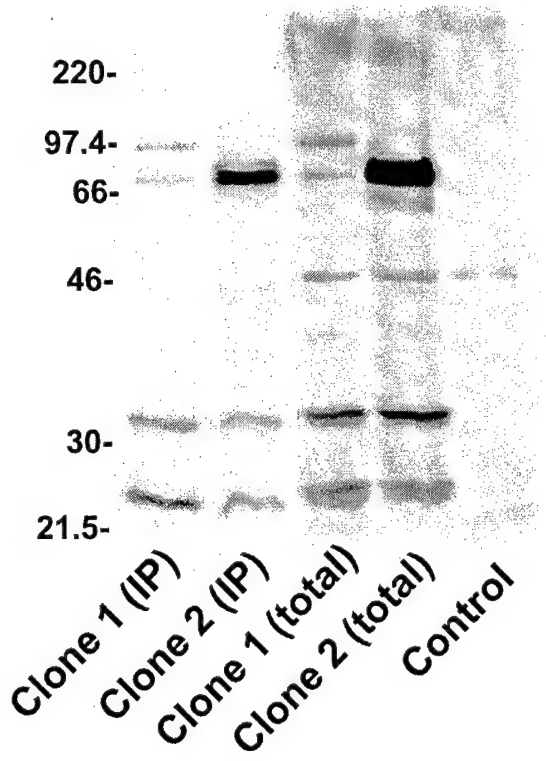

Figure 7. In vitro Transcription/ Translation and Immunoprecipitaion of Putative PCR-7 Protease Clones. Plasmids containing the 3.8 kb (Clone 1) and 3.2 kb (Clone 2) inserts were translated in the presence of ^{35}S methionine using a coupled reticulocyte lysate transcription/translation system (Promega TNT). Aliquots of the translation products were immunoprecipitated with rabbit polyclonal antisera to the GST-PCR-7 fusion protein and electrophoresed on a 10% SDS polyacrylamide gel. 'Control' indicates translation mix less template.

Using a coupled reticulocyte lysate transcription/translation system in the presence of ^{35}S methionine, both clones expressed proteins of approximately 24, 33 and 70 kDa while the 3.8 kb clone expressed a fourth protein of about 80 kDa (Fig. 7). The nature of the 70 and 80 kDa bands is unclear. All were also immunoprecipitated by the antiserum raised against the fusion protein. However, in both MCF-7 cells and trophoblast, the major protein band seen on western blot analysis using this same antibody had an apparent MW of approximately 33 kDa under both reducing and non-reducing conditions (Fig. 8).

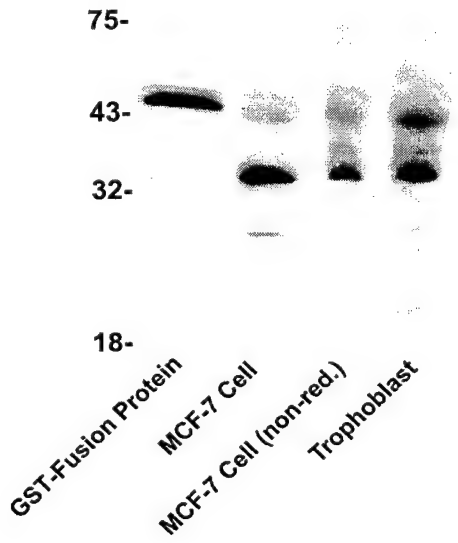

Figure 8. Western Blot Analysis of PCR-7 Protease in MCF-7 Cells and Trophoblast. Monolayers of MCF-7 cells were harvested by scraping, washed with PBS and lysed in SDS gel loading buffer in the presence or absence of β-mercaptoethanol (as indicated). Trophoblasts were isolated from term placenta and cultured for 48 hours before harvesting and lysis under reducing conditions. Aliquots were electrophoresed on a 12% SDS polyacrylamide gel, transferred to nitrocellulose and incubated with a 1-200 dilution of rabbit antibody to the GST-PCR7 fusion protein. Purified GST-PCR7 fusion protein was used as a control. Immune complexes were detected with ^{125}I-Protein A.

MCF-7 cells were surface labeled with [^{3}H]-diisopropylfluorophosphate (DIFP) and immunoprecipitated with antibody to the GST-PCR-7 fusion protein. When total proteins were analyzed by SDS-polyacrylamide gel electrophoresis two labeled bands of approximately 32 and 70 kDa respectively were detected (Fig. 9). Pretreatment of the cells with O-tetradecanoyl-phorbol-13-acetate (TPA), which has previously been shown to stimulate the release of TGFα in MCF-7 cells in the presence of estradiol (Yavelow et al, 1997), had no apparent effect on the intensity of either band. Our failure to immunoprecipitate the ^{3}H-DIFP labeled PCR-7 protease in this preliminary experiment is disturbing as it might indicate that the protein is not on the cell surface. However, it is likely that the labeled bands represent mixtures of several cell surface serine proteases of similar sizes, of which the PCR-7 protease is only a very small fraction. Similarly, this same level of antibody failed to significantly inhibit release of TGFα (Fig. 4). We suspect that not enough specific antibody was present in either of these experiments. Both experiments will be repeated with higher levels of affinity purified antibody to the GST-PCR-7 fusion protein. This will be accomplished by affinity chromatography on immobilized fusion protein. After elution of the antibody from the matrix with the appropriate chaotropic buffer, specific antibody will be further purified by affinity chromatography on immobilized GST.

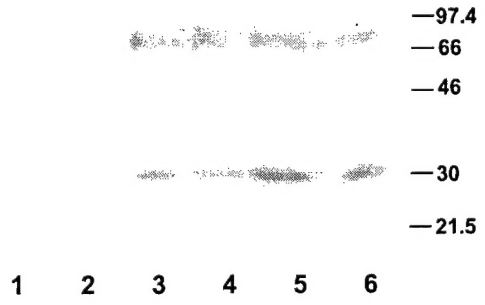

Figure 9. MCF-7 Cells Contain a Cell Surface Protease Which Reacts With ^{3}H DIFP. MCF-7 cells (two ca. 75% confluent T-25 flasks per experiment) were washed with serum-free medium and incubated for 30 min in the absence (lane 1) or presence (lane 2) of 50 ng/ml TPA. ^{3}H DIFP (12.5 μCi/ml, 8.4 μCi/mmol) was added to each flask and the incubation was allowed to continue an additional 60 min. Cells were washed with PBS, lysed in buffer A by passing repeatedly through a 27 1/2 gauge needle and spun for 5 min. in a microfuge at 4° to remove cell debris. After preclearing the cell lysates with pre-immune rabbit IgG and protein A agarose, the supernatants were incubated with 5 μl of specific IgG (24.5 μg/ml) for 2 hours. Immune complexes were collected with protein A agarose, washed 6 times with buffer A, heated for 5 min at 95° C and electrophoresed on a 12% SDS polyacrylamide gel. Aliquots of cell lysates before and after immunoprecipitation were also electrophoresed. After treatment with en^{3}hance, the gel was dried and exposed to x-ray film for 15 days. Lanes 1 and 2, immunoprecipitates; lanes 3 and 4, lysates after immunoprecipitation; lanes 5 and 6, lysates before immunoprecipitation. Lanes 2, 4, and 6 represent samples treated with TPA.

CONCLUSIONS

1. Degenerate oligonucleotides based on the conserved sequences about the $his^{57}$and ser^{195} residues in mammalian serine proteases were used to clone a potential TGFα-releasing serine protease (PCR-7) from an MCF-7 breast cancer cell cDNA library.

2. Northern blot analysis, using a PCR-7 cDNA probe, shows the presence of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells. Similar mRNA species were found in normal breast tissue, trophoblast, and proliferative phase uterine glandular epithelium but not in human U373MG glioma or HepG2 hepatoma cells. In MCF-7 cells, expression of the PCR-7 mRNA was not significantly stimulated by estradiol or TGFα, but was stimulated by IL-6 (200%) and IL-1 (300%).

3. A GST fusion protein constructed from a PCR-7 cDNA sequence was used to raise antibodies in rabbits. On western blot analysis, the antibody recognized a 33 kDa polypeptide in trophoblast and MCF-7 cells under both reducing and non-reducing conditions. This antibody inhibited TPA-induced TGFα release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera.

4. Because of the high degree of sequence homology with known membrane-bound serine proteases paticularly, enterokinase, kininogen, hepsin and prostasin, and because antibodies to a PCR-7-GST fusion protein inhibit TGFα, we believe the PCR-7 sequence to be a membrane-bound TGFα releasing enzyme.

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